Journal of Cellular Physiology

Effects of mild aerobic exercise training on the diaphragm in mdx mice

Journal:	*Journal of Cellular Physiology*
Manuscript ID	JCP-16-0268
Wiley - Manuscript type:	Original Research Article
Date Submitted by the Author:	09-Jun-2016
Complete List of Authors:	Morici, Giuseppe; Universita degli Studi di Palermo, Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC); Frinchi, Monica; Universita degli Studi di Palermo, Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC) Pitruzzella, Alessandro; Universita degli Studi di Palermo, Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC); Istituto Euromediterraneo di Scienza e Tecnologia Di Liberto, Valentina; Universita degli Studi di Palermo, Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC) Barone, Rosario; University of Palermo Pace, Andrea; University of Palermo Dpt. STEBICEF, STEBICEF Di Felice, Valentina; University of Palermo, Department of Experimental Medicine Belluardo, Natale; Università degli Studi di Palermo, Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC) Cappello, Francesco; University of Palermo, Mudò, Giuseppa; Universita degli studi di Palermo, Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC) Bonsignore, Maria ; Consiglio Nazionale delle Ricerche (CNR), Istituto di Biomedicina e Immunologia Molecolare (IBIM); Universita degli Studi di Palermo , Dipartimento Biomedico di Medicina Interna e Specialistica (DiBiMIS)
Key Words:	Duchenne musclular dystrophy, endurance training, chaperonin, stress markers

Effects of mild aerobic exercise training on the diaphragm in *mdx* mice

Giuseppe Morici[1,2], Monica Frinchi[1], Alessandro Pitruzzella[1,3], Valentina Di Liberto[1], Rosario Barone[1,3], Andrea Pace[3,4], Valentina Di Felice[1,3], Natale Belluardo[1], Francesco Cappello[1,3], Giuseppa Mudò[1§], and Maria R Bonsignore[2,5§]

[1]Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche (BioNeC), University of Palermo, Palermo, Italy; [2]Istituto di Biomedicina e Immunologia Molecolare (IBIM), Consiglio Nazionale delle Ricerche (CNR), Palermo, Italy; [3]Istituto Euro-Mediterraneo di Scienza e Tecnologia, Palermo, Italy; [4]Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari (STEMBIO) - University of Palermo, Palermo, Italy; [5]Dipartimento Biomedico di Medicina Interna e Specialistica (DiBiMIS), University of Palermo, Palermo, Italy.

§ Coauthor senior

Running head: Diaphragm regeneration after mild training in *mdx* mice (59)

Author contribution: G Morici and MR Bonsignore conceived the study, obtained funding, contributed to data analysis and interpretation, and wrote the final manuscript. M Frinchi, V Di Liberto, A Pitruzzella, V Di Felice, R Barone, and A Pace performed the experiments and sample analysis, N Belluardo, G Mudò, F. Cappello, and MR Bonsignore contributed to data analysis and interpretation.

Body of text: 2431 words

Corresponding author:
Dr Giuseppe Morici, MD
Dipartimento di Biomedicina e Neuroscienze Cliniche (BioNeC), Sezione di Fisiologia
Corso Tukory 129, Palermo, 90134 Italy
Email: giuseppe.morici@unipa.it

Abstract (234)

Mild endurance exercise training positively affects limb skeletal muscle in the *mdx* mice model of Duchenne Muscular Dystrophy (DMD). However, few and controversial data are available on the effects of mild exercise training on the diaphragm of *mdx* mice. The diaphragm was examined in *mdx* (C57BL/10ScSn-Dmdmdx) mice, and in wild type (WT, C57BL/10ScSc) mice either under sedentary conditions (*mdx*-SD, WT-SD) or during mild exercise training (*mdx*-EX, WT-EX). At baseline, and after 30 and 45 days of training (5 d/wk for 6 weeks), diaphragm muscle morphology and Cx39 protein were assessed. In addition, tissue levels of the chaperonin Hsp60 were measured at the same time points in gastrocnemius, quadriceps and diaphragm in each experimental group. Although morphological analysis showed unchanged total area of necrosis/regeneration in the diaphragm after training, there was a trend for regeneration areas to be larger than necrosis areas in the diaphragm of *mdx*-EX as compared to *mdx*-SD mice. However, the levels of Cx39 protein, a marker associated with active degeneration-regeneration process in damaged muscle were similar in the diaphragm of *mdx*-EX and *mdx*-SD mice. The diaphragm, but not limb muscles, of both trained and sedentary *mdx* mice showed decreased Hsp60 expression at 45 days, suggesting exhaustion of potentially protective mechanisms in the diaphragm similar to previous findings in lung epithelium. Compared to the positive effects of exercise training previously observed in limb skeletal muscles, the diaphragm showed little change after training.

Keywords: Duchenne muscular dystrophy, endurance training, chaperonin, stress markers

Introduction

Duchenne muscular dystrophy (DMD) is a X-linked muscle disease affecting 1:3500 newborn boys (Van Putten et al., 2012), and characterized by a defect in the sub-sarcolemmal protein dystrophin which leads to membrane fragility, muscle necrosis, motor weakness, myofiber death and replacement of skeletal muscle by fibrous and fatty connective tissue (Matthews et al., 1995). In the course of DMD, chronic respiratory insufficiency inevitably develops due to primary loss of inspiratory and expiratory muscle strength (Biggar, 2006).

In the last decade, new strategies have been explored in order to reduce the muscle wasting associated to Duchenne Muscular Dystrophy (DMD). Besides the replacement of functional dystrophin by cell transplantation, and genetic or molecular interventions (Odom et al., 2007; Farini et al., 2009), or recently developed pharmaceutical agents that might slow muscle loss (Bengtsson et al., 2016; De Arcangelis et al., 2016), low-intensity exercise training has been proposed in DMD patients to slow the progression of muscle damage (Grange and Call, 2007).

Similar to human DMD, the *mdx* mouse lacks dystrophin leading to cycles of muscle degeneration and regeneration (Radley-Crabb et al., 2014), and can be used to assess the effects of training on dystrophic muscle (Grounds et al., 2008). We recently reported that inflammatory-necrotic areas in both gastrocnemius and quadriceps muscles of *mdx* mice significantly decreased following low-intensity endurance exercise training for 6 weeks (Frinchi et al., 2014). In more detail, significant recovery of damaged skeletal muscle was observed (Belluardo et al., 2005; Frinchi et al., 2014), and reduced cell degeneration in *mdx* muscles was associated with modulation of proteins involved in oxidative stress defense (Fontana et al., 2015). The majority of studies on the effects of

low-intensity or voluntary exercise reported protective effects of training on skeletal muscles of *mdx* mice (Baltgalvis et al., 2012; Call et al., 2010; Frinchi et al. 2014; Gordon et al., 2014; Hayes and Williams, 1996) (Table 1).

In the diaphragm, degenerative changes similar to those of human DMD where shown in sedentary *mdx* mice (Petrof et al., 1993; Stedman et al., 1991). Few studies have examined the effects of exercise training on the diaphragm, reporting variable and mostly negative results (Dupont-Versteegden et al., 1994; Selsby et al., 2013) (Table 1).

Aim of this study was to assess the effects of low-intensity endurance exercise training on the diaphragm of *mdx* mice. In the diaphragm of sedentary and low-intensity endurance exercise trained *mdx* mice, we studied histopathology and the expression of connexin39 (Cx39), a specific gene involved in skeletal muscle regeneration and considered as a quantitative marker of muscle regeneration [Belluardo et al., 2005; Frinchi et al., 2014]. Moreover, we assessed the role of the chaperonin Hsp60, a mitochondrial molecule essential for cell metabolism and survival, in both limb and diaphragm muscles during mild aerobic training in *mdx* and control mice.

Materials and methods

Animals and animal care

The present study was performed using the diaphragm samples of mice used in our previous work on limb skeletal muscles (Frinchi et al., 2014). Therefore, muscle samples used in the present study were obtained from the following experimental groups: 8 week-old male *mdx* mice (C57BL/10ScSn-Dmdmdx/J Jackson Laboratories), and C57/BL wild type (WT) mice (C57BL/10ScSn, Harlan, Italy) were randomly

assigned to sedentary (SD) (MDX-SD n=17; WT- SD n=19) or trained (EX) (MDX-EX n=14; WT-EX n=16) groups.

Animals were trained using a motorized rotating treadmill (Rota-Rod; Ugo Basile, Biological Research Apparatus, Comerio Varese, Italy), in a protected environment and in the same room where the mice were housed (Frinchi et al., 2014). Mice ran 5 days/week for 4 weeks at progressively increasing loads (Table 2) (Barone et al., 2013). Trained and sedentary WT and *mdx* mice were sacrificed by an overdose of chloral hydrate anesthesia at time 0, 30 and 45 days. Procedures involving animals and their care were conducted in conformity with the Italian institutional guidelines (D.L. 116, G.U., suppl. 40, February 18, 1992).

Histological examination

The diaphragm muscle was excised under stereomicroscopy, covered with OCT and frozen in isopentane precooled in liquid nitrogen, and stored at −80°C until use. Diaphragm sections (10 μm) were cut at −20°C and thawed onto 3-aminopropyl-ethoxysilane-coated slides. From each muscle, 10 sections were collected by systematically sampling every 5 sections. Frozen muscle sections were directly fixed in 4% paraformaldehyde and stained with hematoxylin-eosin and then dehydrated with ethanol and xylene, mounted with Entellan (Merck, Darmstadt, Germany), examined by stereomicroscope (Leica Microsystems Imaging solutions Ltd, Cambridge, UK) and acquired by digital camera (Leica, DFC Camera). Necrosis/regeneration areas in the muscle section were identified as areas of increased inflammatory infiltration and centrally nucleated fibers, respectively, and measured by ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA,

http://imagej.nih.gov/ij/, 1997–2014). For each muscle section, the total area of muscle section was measured in mm^2. All counts were carried out in a double blind manner.

Western Blotting Analysis

To assess and quantify the recovery of damaged diaphragm muscle in *mdx* mice the Cx39 protein was used as a marker of active necrosis-regeneration process (Frinchi et al., 2014). Hsp60 protein levels was performed on diaphragm, gastrocnemius and quadriceps muscles. The muscles were rapidly dissected and frozen in precooled isopenthane. Half of each sample was homogenized in cold buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% triton, 0.5% SDS, H_2O and protease inhibitor cocktail (P8340, Sigma-Aldrich S.r.l., Milan, Italy). The homogenate was left on ice for 30 min and then centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatants were stored at -80°C and aliquots were taken for protein determination (Lowry et al., 1951). The samples with 30 µg of protein and mol.wt. markers (161-0375, Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy), were run on 8% and/or 12% polyacrylamide gel at 100 V and electrophoretically transferred onto nitrocellulose membrane (Hybond-C-extra, GE Healthcare, formerly Amersham, Europe GmbH –Milan, Italy). Following 1 h of incubation in blocking buffer (1x TBS, 0.1% Tween-20, 5% w/v nonfat dry milk) the membrane was incubated with gentle shaking overnight at +4°C in the same buffer with rabbit anti-Cx39 affinity purified antibodies (1:1000) (SC104847, M-13 Santa Cruz Biotechnology), mouse monoclonal antibody anti-Hsp60 (1:5000) (ab13532, Abcam), or rabbit polyclonal antibody anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000) (ADI905784, Enzo Life Sciences). After washing, the membrane was incubated for 1 h at room temperature with anti-rabbit IgG horseradish peroxidase-

conjugated diluted 1:8000 (Sc 2004, Santa Cruz Biotechnology), or anti-mouse HRP-conjugated secondary antibody diluited 1:5000 (NA931, Amersham Biosciences) and band was visualized by chemiluminescence (ECL, GE Healthcare, Amersham). The blot was exposed to autoradiography film (Amersham Hyperfilm ECL; 28-9068-36), developed in Kodak D19 developer and fixer, and the densitometric evaluation of bands was performed by measuring the optical density (O.D.) using NIH ImageJ software. Results were expressed as arbitrary units.

Statistical analyses

Data are expressed as mean ± SD and unpaired t-test or one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons were used as appropriate analysis. All statistical analyses were performed using the GraphPad PrismTM 4.0 program (GraphPad Software Inc., San Diego, California, USA) or Statview 5.0 for Windows. The level of statistical significance was set at $p<0.05$.

Results

Histopathology of diaphragm and quantification of diaphragm injury

The effects of low-intensity endurance exercise on pathology progression in the diaphragm of *mdx* mice were assessed at 30 and 45 days from training, similar to the previous study on gastrocnemius and quadriceps muscles of *mdx* mice by our group (Frinchi et al., 2014). No inflammatory-necrotic areas or regeneration areas were observed in the diaphragm of WT mice, irrespective of training (Fig. 1A). In the diaphragm of *mdx* mice, the total area of necrosis-regeneration did not show any significant difference between SD and EX mice (Fig. 1B and C). However, when the

inflammatory-necrotic and regeneration areas were separately evaluated in the context of the same total areas, *mdx*-EX mice showed a large area of active regeneration, identified by centrally nucleated cells with large cytoplasm, and a very small area of necrosis (Fig. 1C). Conversely, *mdx*-SD mice showed a large area of necrosis and a small area of regeneration (Fig. 1C) at both 30 and 45 days.

To assess and quantify the recovery of damaged muscle in *mdx* mice the Cx39 protein levels were used as a marker of active necrosis-regeneration process (Frinchi et al., 2014). Analysis of Cx39 protein showed undetectable levels of Cx39 levels in WT mice irrespective of training (Fig.1D). As expected Cx39 was found up-regulated in *mdx*-SD mice, reflecting active muscle necrosis-regenerating processes, and the comparative analysis of Cx39 protein levels at either 30 or 45 days of training showed nonsignificant difference between *mdx*-SD and *mdx*-EX mice (Fig. 1D). Therefore, training did not affect the Cx39 levels in the diaphragm of *mdx* mice in contrast with the decreased Cx39 levels found in the gastrocnemius and quadriceps muscles of trained *mdx* mice, in our previous work (Frinchi et al., 2014).

Hsp60 protein levels

Lysates of diaphragm, gastrocnemius and quadriceps muscles were analysed by western blotting analysis in all experimental groups. No differences were found among groups at 0, 30 and 45 days (Fig. 2). However, Hsp60 levels in the diaphragm muscle were significantly lower at 45 days in *mdx* than WT mice, irrespective of trained or sedentary status ($p<0.05$).

Discussion

The aim of this study was to assess the response of the diaphragm to low-intensity endurance training. Although morphological analysis showed unchanged total area of necrosis/regeneration in the diaphragm after training, there was a trend for regeneration areas to be larger than necrosis areas in the diaphragm of *mdx*-EX as compared to *mdx*-SD mice. However, the levels of Cx39 protein, a marker associated with active degeneration-regeneration process in damaged muscle (Frinchi et al., 2014), were not significantly different in the diaphragm of mdx-EX and mdx-SD mice. The diaphragm, but not limb muscles, of both trained and sedentary *mdx* mice showed decreased Hsp60 expression at 45 days, suggesting exhaustion of potentially protective mechanisms in the diaphragm similar to previous findings in lung epithelium (Morici et al., 2014).

In *mdx* mice, mild endurance training was associated with decreased Cx39 expression in limb skeletal muscles (Frinchi et al., 2014), and this reduction correlated with significant muscle regeneration. The discrepancy between increased regeneration and similar Cx39 protein levels in the diaphragm of exercised and sedentary *mdx* mice could be explained by occurrence of inflammation in regenerating areas, suggesting coexistence of regenerating fibers and necrosis process. We speculate that this behaviour might be peculiar to the diaphragm muscle and possibly correlated to its continuous activity. It is known that Cx39 can be detected in both necrosis and regeneration areas. However, the similar levels of Cx39 in mdx-SD and mdx-EX mice may indicate that mild training was not detrimental in the diaphragm muscle.

Table 1 summarizes the results of the main studies on the effects of exercise training on skeletal muscle and diaphragm in *mdx* mice. The large majority of studies reported

positive effects of exercise on limb skeletal muscles, irrespective of the training protocol used. Table 1 also shows that the response to training in the *mdx* diaphragm is poorly defined due to the low number of available studies and their mostly negative results. On the clinical side, one study reported that inspiratory muscle training improved respiratory muscle function in patients with early stage DMD (Wanke et al., 1994). The only positive results in *mdx* mice were reported by Dupont-Versteegden and coworkers (1994), who found improved diaphragmatic function after extensive running. In sedentary *mdx* mice, morphological changes in the diaphragm were very similar to those observed in DMD patients (Stedman et al., 1991). A prolonged increase in airway resistance obtained by tracheal banding in *mdx* mice was associated with evidence of increased fiber regeneration and unchanged progression of dystrophy compared to control (Krupnick et al., 2003). Chronic volitional running was associated with decreased tension of the diaphragm in *mdx* mice (Selsby et al., 2013). More recently, Barbin and coworkers (2016) in swimming-trained 11-month old mice found increased fibrosis of the diaphragm and increased activity of the fibrosis marker MMP-2. Differently from the latter data, no evidence for diaphragmatic fibrosis was found after 6 weeks of mild running training in our mice. Indeed, expression of Cx39, increased with age in the diaphragm of *mdx* mice irrespective of training, suggesting that in the diaphragm the functional stress under sedentary conditions might be worse than in other skeletal muscle groups and mild training could not exert adequate beneficial effects as in limb muscles.

The expression of Hsp60, a chaperonin involved in cell and tissue homeostasis, in limb muscles and diaphragm can provide a further insight into the mechanisms of muscle damage in *mdx* mice. In wild type mice after 45 days of training, increased in

soleus muscles during endurance training (Barone et al., 2016). In airway epithelium of *mdx* mice, we previously reported that Hsp60 expression initially increased (30 days), then decreased (45 days) irrespective of training (Morici et al., 2016); the fall in Hsp60 expression at 45 days was associated with increased apoptosis of epithelial cells, suggesting a protective role of Hsp60 in the lung. Therefore, we decided to assess the time course of Hsp60 in skeletal muscles and diaphragm of *mdx* mice during training. No difference in Hsp60 expression was found between wild type and *mdx* mice at any time in either gastrocnemius or quadriceps (Fig. 2). Instead, the diaphragm showed decreased Hsp60 expression in *mdx* mice at 45 days irrespective of training, with a time course similar to our observations in airway epithelial cells (Fig. 2). We speculate that the ability to maintain a high level of Hsp60 in gastrocnemius and quadriceps might contribute to the positive effects of exercise training in limb muscles, while the decreased levels found in the diaphragm might contribute to the lack of major positive effects of mild exercise in this district. Recently, Gehrig and co-workers (2012) reported that increased expression of Hsp72 slowed muscle damage in the diaphragm of *mdx* mice by preventing inflammation and cellular stress. Hsp72 positively modulates the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), a protein responsible for calcium reuptake which showed a progressive decrease over time in the diaphragm of *mdx* mice (Gehrig et al., 2012), similar to the changes we observed in Hsp60. These data suggest a possible major role of chaperone proteins in the pathophysiology of muscle damage in *mdx* mice.

In conclusion, our data indicate that after mild aerobic training little benefit is evident in the diaphragm of *mdx* mice as compared to limb muscles and we speculate that the

reduced positive effect of training in the diaphragm of *mdx* mice could be attributed to high rate of muscle degeneration insufficiently counteracted by repair mechanisms.

Acknowledgements

This work was partly supported by the Euro-Mediterranean Institute of Science and Technology (FC, VDF and A Pitruzzella) and the University of Palermo (A Pace, FC, MF, G Morici, VDL, MRB, NB, RB, G Mudò) funds. Part of this work was carried out using instruments provided by the Euro-Mediterranean Institute of Science and Technology and funded with the Italian National Operational Programme for Research and Competitiveness 2007-2013 grant (Project code: PONa3_00210, European Regional Development Fund).

References

Baltgalvis KA, Call JA, Cochrane GD, Laker RC, Yan Z, Lowe DA. 2012. Exercise training improves plantar flexor muscle function in mdx mice. Med Sci Sports Exerc 44:1671-1679.

Barbin IC, Pereira JA, Bersan Rovere M, de Oliveira Moreira D, Marques MJ, Santo Neto H. 2016. Diaphragm degeneration and cardiac structure in mdx mouse: potential clinical implications for Duchenne muscular dystrophy. J Anat 228:784-791.

Barone R, Macaluso F, Catanese P, Marino Gammazza A, Rizzuto L, Marozzi P, Lo Giudice G, Stampone T, Cappello F, Morici G, Zummo G, Farina F, Di Felice V. Endurance exercise and conjugated linoleic acid (CLA) supplementation up-regulate CYP17A1 and stimulate testosterone biosynthesis. PLoS One 2013 Nov 5;8(11):e79686.

Barone R, Macaluso F, Sangiorgi C, Campanella C, Marino Gammazza A, Moresi V, Coletti D, Conway de Macario E, Macario AJ, Cappello F, Adamo S, Farina F, Zummo G, Di Felice V. 2016. Skeletal muscle Heat shock protein 60 increases after endurance training and induces peroxisome proliferator-activated receptor gamma coactivator 1 α1 expression. Sci Rep 6:19781.

Belluardo N, Trovato-Salinaro A, Mudò G, Condorelli DF. 2005. Expression of the rat connexin 39 (rCx39) gene in myoblasts and myotubes in developing and regenerating skeletal muscles: an in situ hybridization study. Cell Tissue Res 320: 299-310.

Bengtsson NE, Seto JT, Hall JK, Chamberlain JS, Odom GL. 2016. Progress and prospects of gene therapy clinical trials for the muscular dystrophies. Hum Mol Genet 25(R1):R9-R17

Biggar WD. 2006. Duchenne muscular dystrophy. Pediatr Rev 27: 83-88.

Call JA, McKeehen JN, Novotny SA, Lowe DA. 2010. Progressive resistance voluntary wheel running in the mdx mouse. Muscle Nerve 42: 871-880.

De Arcangelis V, Strimpakos G, Gabanella F, Corbi N, Luvisetto S, Magrelli A, Onori A, Passananti C, Pisani C, Rome S, Severini C, Naro F, Mattei E, Di Certo MG, Monaco L. 2016. Pathways implicated in Tadalafil amelioration of Duchenne Muscular Dystrophy. J Cell Physiol 231: 224-232.

Dupont-Versteegden EE, McCarter RJ, Katz MS. 1994. Voluntary exercise decreases progression of muscular dystrophy in diaphragm of mdx mice. J Appl Physiol 77: 1736-1741.

Farini A, Razini P, Erratico S, Torrente Y, Meregalli M. 2009. Cell based therapy for Duchenne muscular dystrophy. J Cell Physiol 221: 526-534.

Fontana S, Schillaci O, Frinchi M, Giallombardo M, Morici G, Di Liberto V, Alessandro R, De Leo G, Perciavalle V, Belluardo N, Mudò G. 2015. Reduction in mdx mouse muscle degeneration by low-intensity endurance exercise: a proteomic analysis in quadriceps muscle of exercised compared with sedentary mdx mice. Biosci Rep 35(3), pii: e00213. doi: 10.1042/BSR20150013.

Frinchi M, Macaluso F, Licciardi A, Perciavalle V, Coco M, Belluardo N, Morici G, Mudò G. 2014. Recovery of damaged skeletal muscle in mdx mice through low-intensity endurance exercise. Int J Sports Med 35:19-27.

Gehrig SM, van der Poel C, Sayer TA, Schertzer JD, Henstridge DC, Church JE, Lamon S, Russell AP, Davies KE, Febbraio MA, Lynch GS. 2012. Hsp72 preserves muscle function and slows progression of severe muscular dystrophy. Nature 484: 394-398

Gordon BS, Lowe DA, Kostek MC. 2014. Exercise increases utrophin protein expression in the mdx mouse model of Duchenne muscular dystrophy. Muscle Nerve 49: 915-918.

Grange RW, Call JA. 2007. Recommendations to define exercise prescription for Duchenne muscular dystrophy. Exerc Sport Sci Rev 35: 12-17.

Grounds MD, Radley HG, Lynch GS, Nagaraju K, De Luca A. 2008. Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. Neurobiol Dis 31: 1-19.

Hayes A, Williams DA. 1996. Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle. J Appl Physiol 80: 670-679.

Kaczor JJ, Hall JE, Payne E, Tarnopolsky MA. 2007. Low intensity training decreases markers of oxidative stress in skeletal muscle of mdx mice. Free Radic Biol Med 43:145-154.

Krupnick AS, Zhu J, Nguyen T, Kreisel D, Balsara KR, Lankford EB, Clark CC, Levine S, Stedman HH, Shrager JB. 2003. Inspiratory loading does not accelerate dystrophy in mdx mouse diaphragm: implications for regenerative therapy. J Appl Physiol 94: 411-419.

Landisch RM, Kosir AM, Nelson SA, Baltgalvis KA, Lowe DA. 2008. Adaptive and nonadaptive responses to voluntary wheel running by mdx mice. Muscle Nerve 38: 1290-1303.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Matthews PM, Benjamin D, Van Bakel I, Squier MV, Nicholson LV, Sewry C, Barnes PR, Hopkin J, Brown R, Hilton-Jones D, Boyd Y, Karpati G, Brown GK, Craig IW. 1995. Muscle X-inactivation patterns and dystrophin expression in Duchenne muscular dystrophy carriers. Neuromuscul Disord 5: 209-220.

Morici G, Rappa F, Cappello F, Pace E, Pace A, Mudò G, Crescimanno G, Belluardo N, Bonsignore MR. 2016. Lack of dystrophin affects bronchial epithelium in mdx mice. J Cell Physiol doi: 10.1002/jcp.25339.

Odom GL, Gregorevic P, Chamberlain JS. 2007. Viral-mediated gene therapy for the muscular dystrophies: successes, limitations and recent advances. Biochim Biophys Acta 1772(2):243-262.

Okano T, Yoshida K, Nakamura A, Sasazawa F, Oide T, Takeda S, Ikeda S. 2005. Chronic exercise accelerates the degeneration-regeneration cycle and downregulates insulin-like growth factor-1 in muscle of mdx mice. Muscle Nerve 32: 191-199.

Petrof BJ, Stedman HH, Shrager JB, Eby J, Sweeney HL, Kelly AM. 1993. Adaptations in myosin heavy chain expression and contractile function in dystrophic mouse diaphragm. Am J Physiol 265: C834-C841.

Radley-Crabb HG, Marini JC, Sosa HA, Castillo LI, Grounds MD, Fiorotto ML. 2014. Dystropathology increases energy expenditure and protein turnover in the mdx mouse model of duchenne muscular dystrophy. PLoS One 9(2): e89277.

Selsby JT, Acosta P, Sleeper MM, Barton ER, Sweeney HL. 2013. Long-term wheel running compromises diaphragm function but improves cardiac and plantarflexor function in the mdx mouse. J Appl Physiol 115: 660-666.

Smythe GM, White JD. 2011. Voluntary wheel running in dystrophin-deficient (mdx) mice: Relationships between exercise parameters and exacerbation of the dystrophic phenotype. Version 3. PLoS Curr 3: RRN1295.

Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B, Narusawa M, Leferovich JM, Sladky JT, Kelly AM. 1991. The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. Nature 352: 536-539.

Van Putten M, Hulsker M, Nadarajah VD, van Heiningen SH, van Huizen E, van Iterson M, Admiraal P, Messemaker T, den Dunnen JT, 't Hoen PA, Aartsma-Rus A. 2012. The effects of low levels of dystrophin on mouse muscle function and pathology. PLoS One 7(2):e31937.

Wanke T, Toifl K, Merkle M, Formanek D, Lahrmann H, Zwick H. 1994. Inspiratory muscle training in patients with Duchenne muscular dystrophy. Chest 105: 475-482.

Figure legends

Figure 1. Diaphragm muscle sections from wild type (WT) and *mdx* mice stained with haematoxylin-eosin, and related morphometric analysis. Panel A: diaphragm muscle sections from wild type sedentary (WT-SD) and exercised (WT-EX) mice showing no areas of necrosis or regeneration. Panel B: diaphragm muscle sections showing areas of necrosis and regeneration (sampled high magnification areas) in *mdx*-sedentary (MDX-SD) and *mdx*-exercised (MDX-EX) mice at 30 days of training.

Panel C: quantitative analysis of necrosis and regeneration areas in *mdx* mice, measured in mm^2 and expressed as percentage of total mm^2 of muscle section area. Although the total area of necrosis-regeneration was similar in *mdx*-SD and *mdx*-EX mice, necrosis area was significantly reduced and regeneration area (identified by centrally nucleated cells with large cytoplasm) was higher in the diaphragm of *mdx*-EX mice as compared to *mdx*-SD mice.

Panels D: western blotting analysis of Cx39 protein levels in diaphragm muscle of sedentary (0 days) and exercised (45 days) WT mice, and of sedentary and exercised *mdx* mice at 0, 30 and 45 days. Cx39 protein was undetectable in the diaphragm of sedentary and exercised WT mice, whereas was high expressed in *mdx*-EX and *mdx*-SD mice but did not show difference between two groups. Group data reported as means ± S.E.M, * $p<0.02$ between sedentary and exercised *mdx* mice. Scale bars: low magnification 200 μm; high magnification 50 μm.

Figure 2. Hsp60 protein levels decreased in the diaphragm muscle of *mdx*-SD and *mdx*-EX mice. Representative western blots (A) and relative expression levels (B) of Hsp60 in quadriceps, gastrocnemius and diaphragm muscles of wild type sedentary (WT-SD),

wild type trained (WT-EX), *mdx*-sedentary (*mdx*-SD) and *mdx*-trained (*mdx*-EX) mice at 0, 30 and 45 days. GAPDH was used as the loading control. Group data reported as means ± SD. * significantly different ($P < 0.05$).

Training duration and type	Limb muscles	Diaphragm
10-13 mo, voluntary wheel running		Exercise increased active tension in *mdx* diaphragm by ~ 30%
16 wk, voluntary wheel running	Increased absolute muscle mass and twitch and tetanic tension of the *mdx* soleus; higher resistance to fatigue of the extensor digitorum longus in trained *mdx* compared to sedentary *mdx* mice	
, 18 and 24 wk, increased work of the diaphragm in vivo (tracheal banding)		Unchanged progression of dystrophy compared to control *mdx* mice; centrally nucleated fibers evident in the diaphragms of banded *mdx* mice
5 and 10 wk, upwards running (7° slope), at 5 and 23 m/min for 60 min, twice/wk	Accelerated myofiber regeneration and decreased release of insulin growth factor-1 (IGF-1) in hindlimb muscles	
8 wk, 30 min/day, twice/wk, low-intensity endurance exercise (treadmill)	Lower levels of markers of oxidative stress in white gastrocnemius	
8 wk, voluntary wheel running	Beneficial adaptations in muscle mass, fiber size, and fiber types in hindlimb muscles	
12 wk, voluntary wheel running; protocol: a) no resistance, b) progressively increasing resistance	Greater grip strength (~22%) and soleus muscle specific tetanic force (26%) in both trained groups compared with sedentary controls	
2 wk, voluntary wheel running	In the quadriceps, fewer running bouts and higher bout distance promoted necrosis and muscle damage	
12 wk, voluntary low-resistance wheel running	Plantaflexors function improved	
One year, volitional wheel running	Limb muscle function largely unaffected, plantaflexors function improved	Impaired function

6 wk, 5 d/wk, low-intensity endurance exercise (motorized Rota-Rod)	Strong reduction of degeneration process in gastrocnemius and quadriceps muscles	
12 wk, voluntary aerobic wheel running	Increased utrophin protein expression in the quadriceps after training	
2 months, swimming (60 min/day, 6 d/wk)		Increased MMP-2 activity and fibrosis

Table 2. Acclimatization (A) and training (T) protocol in WT and *mdx* mice

Week	Session duration (min)	Rotations •min^{-1}	Distance (m)
1 (A)	15	16	48
2 (A)	30	16	96
3 (T)	30	20	120
4 (T)	45	20	180
5 (T)	60	20	240
6 (T)	60	24	288

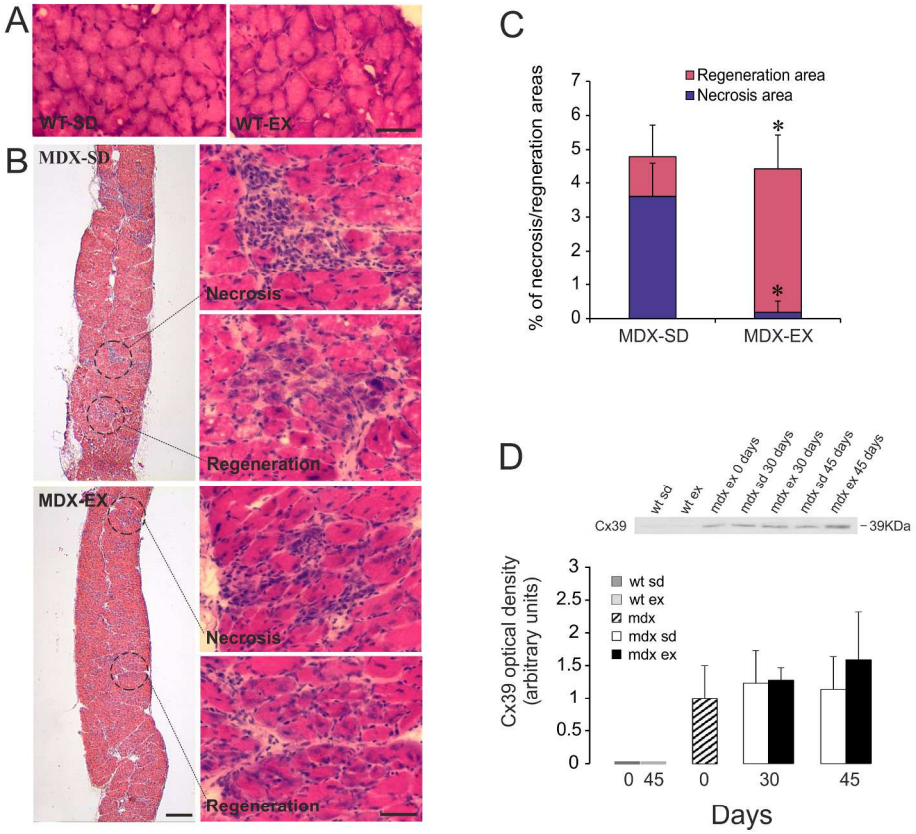

Figure 1. Diaphragm muscle sections from wild type (WT) and mdx mice stained with haematoxylin-eosin, and related morphometric analysis. Panel A: diaphragm muscle sections from wild type sedentary (WT-SD) and exercised (WT-EX) mice showing no areas of necrosis or regeneration. Panel B: diaphragm muscle sections showing areas of necrosis and regeneration (sampled high magnification areas) in mdx-sedentary (MDX-SD) and mdx-exercised (MDX-EX) mice at 30 days of training.
Panel C: quantitative analysis of necrosis and regeneration areas in mdx mice, measured in mm2 and expressed as percentage of total mm2 of muscle section area. Although the total area of necrosis-regeneration was similar in mdx-SD and mdx-EX mice, necrosis area was significantly reduced and regeneration area (identified by centrally nucleated cells with large cytoplasm) was higher in the diaphragm of mdx-EX mice as compared to mdx-SD mice.
Panels D: western blotting analysis of Cx39 protein levels in diaphragm muscle of sedentary (0 days) and exercised (45 days) WT mice, and of sedentary and exercised mdx mice at 0, 30 and 45 days. Cx39 protein was undetectable in the diaphragm of sedentary and exercised WT mice, whereas was high expressed in mdx-EX and mdx-SD mice but did not show difference between two groups. Group data reported as means ± S.E.M, * $p<0.02$ between sedentary and exercised mdx mice. Scale bars: low magnification 200 micron; high magnification 50 micron
237x228mm (300 x 300 DPI)

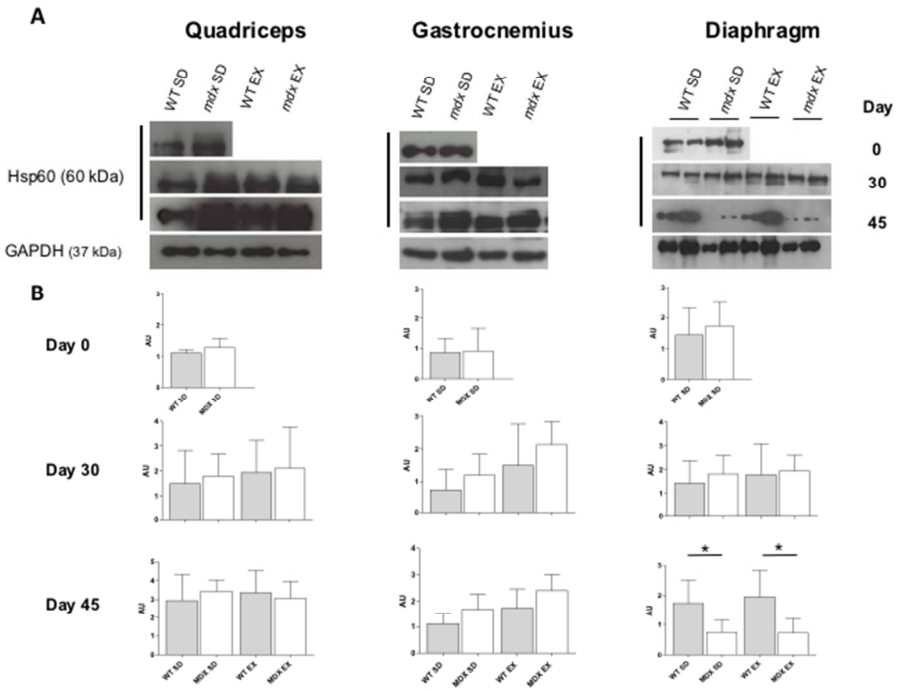

Figure 2. Hsp60 protein levels decreased in the diaphragm muscle of mdx-SD and mdx-EX mice. Representative western blots (A) and relative expression levels (B) of Hsp60 in quadriceps, gastrocnemius and diaphragm muscles of wild type sedentary (WT-SD), wild type trained (WT-EX), mdx-sedentary (mdx-SD) and mdx-trained (mdx-EX) mice at 0, 30 and 45 days. GAPDH was used as the loading control. Group data reported as means ± SD. * significantly different ($P < 0.05$).
254x190mm (72 x 72 DPI)